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Sialyl-Lewis a (SA-Lea) and its structural isomer Sialyl-Lewis X (SA-LeX) carbohydrates are expressed on leukocytes and carcinoma cells and are E-selectin ligands. A SA-Lea mimicking peptide with the sequence DLWDWVVGKPAG was previously identified by us by screening a combinatorial peptide library using a SA-Lea specific monoclonal antibody (MAb) NS19-9. In further studies, peptide DLWDWEVGKPAG was identified using peptide array which showed higher MAb binding affinity as compared to the lead peptide. We demonstrated that a peptide mimics of SA-Lea mediate a 30% and 50% inhibition of neutrophil recruitment in acute inflammatory conditions and lung colonization of tumor cells in an *in vivo* murine tumor model, respectively. Similar effect was achieved in E-selectin deficient mice, where metastases were completely abrogated using our established tumor model. Thus, data demonstrate that E-selectin and carbohydrate ligand, SA-Lea play an important role in inflammatory conditions and most importantly for this project establishing metastases *in vivo*. The peptides described here provide for further elucidation of the fine specificity of oligosaccharide-protein interactions and the role played by carbohydrate tumor-associated antigens in the metastatic process.

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Carbohydrate Mimicking Peptides as Inhibitors of Angiogenesis and Metastasis

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5. Introduction

Carbohydrate structures expressed on the surface of leukocytes and tumor cells mediate specific recognition by cell-surface selectins expressed on cytokine activated endothelial cells (EC) and platelets, underlying inflammation and metastasis, respectively. The intercellular interaction between EC and leukocytes and tumor cells represents a functional prototype of protein-carbohydrate interactions. Carbohydrate ligands such as SA-LeX and SA-Lea are found on the surface of leukocytes and tumor cells, and their receptor, E-selectin, is expressed on activated endothelial cells (1,2). Since both neutrophils and carcinoma cells express ligands for E-selectin, it was attractive to hypothesize that early steps of metastatic colonization (3-5) may be considered equivalent to the initiation of inflammatory process (4-10). An objective of the proposed studies was to explore the possibility that interruption of protein-carbohydrate based adhesion of tumor cells to vascular endothelium reduces significantly metastatic spread in analogy with reduction of neutrophil recruitment in inflammatory conditions.

Specifically, we proposed to use combinatorial libraries to identify structural equivalents or peptide mimotopes of carbohydrate ligands for E-selectin such as SA-Lea and SA-LeX and E-selectin independent adhesion ligand LeY using specific MAbs and to investigate the effect of the identified peptides for their ability to interfere with tumor cell attachment and angiogenesis in vitro using various adhesion assays and metastatic processes in vivo.

6. Body

Combinatorial peptide library. Combinatorial peptide library was used to select peptides mimicking carbohydrate ligands important for inflammation and metastasis such as SA-Lea, SA-LeX and Lewis Y as described in the previous annual reports and Appendix 1 and 2. The peptide DLWDWVVGKPAG mimicking SA-Lea was selected from the family of peptide identified with specific monoclonal antibody MAb, NS19-9 MAb because it contains the consensus motif identified among multiple clones and also it displayed stronger signal in Western blot. The ability of the peptide DLWDWVVGKPAG to compete with the cognate oligosaccharide determinant (SA-Lea-PAA neoglycoprotein) for MAb binding in solution was tested in a competition ELISA. Synthetic peptide competitively inhibited the binding of NA19-9 MAb in dose dependent manner (concentration range from 1 mM to 5 mM), suggesting that the peptide sterically interferes with MAb binding to carbohydrate antigen. The 50% inhibitory concentration (IC_{50}) value of the peptide was calculated at 700 mM implying that the sequence DLWDWVVGKPAG represents a solvent-accessible epitope for MAb and that the peptide and carbohydrate binding sites are overlapping. Control peptide failed to compete with MAb NS 19-9 binding in the concentration range of to 5 mM, indicating that the inhibitory effects of the native sequences are due to specific effects (Appendix 2).

Acute inflammation model in vivo. The accumulation of neutrophils is a characteristic feature of acute and chronic inflammatory disease and early steps in the recruitment of these cells to the site of inflammation, depends upon E-selectin-mediated interaction. Thus, inhibition of neutrophil recruitment *in vivo* is an important test of the ability of potential therapeutics agents to inhibit E-selectin-mediated events. An interaction with another E-selectin carbohydrate ligand, SA-Lea, is not relevant for adhesion of neutrophils, since SA-Lea is not expressed on PMN surface. Nevertheless, because SA-Lea binds to E-selectin, we tested whether administration of a SA-Lea mimicking molecule would diminish the influx of neutrophils into chemically irritated peritoneum *in vivo*. Thus, to assess the bioactivity of the 12-mer peptide D L W D W V V G K P AG mimicking the SA-Lea carbohydrate structure the Zymosan was administered intraperitoneally (i.p.) into mice followed 3 hr later by an intravenous (i.v.) injection of peptide (1 mg/mouse). Neutrophils were harvested by peritoneal lavage and enumerated 1 hr later. 30% of reduction in number of neutrophils in peritoneal lavage fluids was observed upon peptide treatment as

compared to the animals treated with irrelevant peptide, Fig. 1. The results were statistically significant (P<0.001).

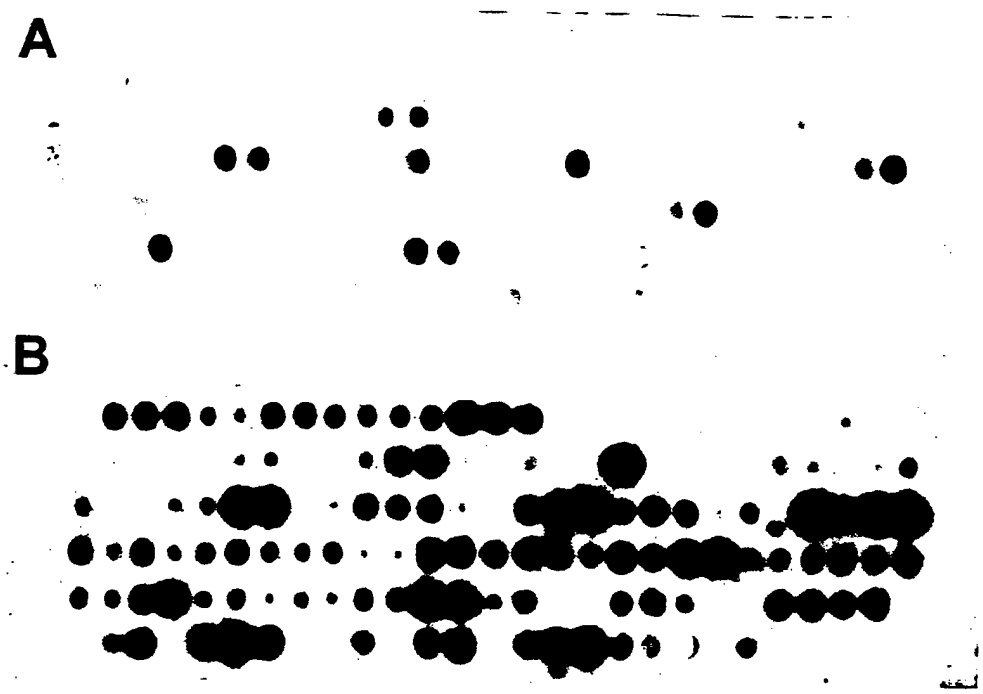

Figure 1. Neutrophil influx upon administration of peptide #4 DLWDWVVGKPAG mimicking SA-Lea carbohydrate in mice with chemically induced peritonitis, #4. Control peptide, C. Results are from 4 experiments (3 mice in each group). A, Neutrophil counts; B, Myeloperoxidase activity in collected neutrophils. Unrelated peptide was administered in control mice. Statistical analysis using nonparametric unpaired t-test gave P values <0.001 and <0.005 for data in A and B, respectively.

To confirm these results, myeloperoxidase (MPO) activity, which is an enzymatic marker for neutrophils (14), was measured spectrophotometrically as an absorbance rate in the homogenates of collected cells. 30% of reduction of enzymatic activity at P<0.005 value was observed in parallel with decreased neutrophil numbers assessed by total neutrophil count (Fig. 1B). These results strongly suggest that the statistically significant reduction in enzyme activity is due to blocking in neutrophil recruitment in mice treated with peptide mimicking E-selectin ligand SA-Lea and that this peptide can inhibit E-selectin function *in vivo*. (Fig 1).

SA-Lea peptide mimic with Higher MAb Binding Affinity. To analyze amino acid residues that are critical for NS19-9 recognition, an array library of 163 peptides was generated by systematic amino acid replacement in which each position of the starting peptide DLWDWVVGKPAG was replaced by other L-amino acids (15) (Appendix 2). In addition, peptides were synthesized with simultaneous incorporation of multiple amino acids or with truncation of specific regions. The ability of MAb NS19-9 to bind substituted peptides within the library was determined after probing of the membrane containing peptide spots with NS19-9 followed by chemiluminescence detection with a peroxidase-labeled anti mouse immunoglobulin G antibody. Spot analysis revealed a distinct pattern of key residues important for binding and,

therefore, sensitive to substitution while other residues tolerated replacement by a variety of amino acids (Fig. 2). Comparison of the signal intensities of the array scan revealed that the critical residues for binding were clearly identified within the N-terminal half of the DLWDWVVGKPAG peptide as determined by the lack of antibody binding to substituted peptides. In contrast, most of the substitutions within the C-terminus were tolerated (amino acids 6 to 12), not influencing MAb binding. These results indicate that the N-terminus is clearly involved in specific interaction with NS19-9.

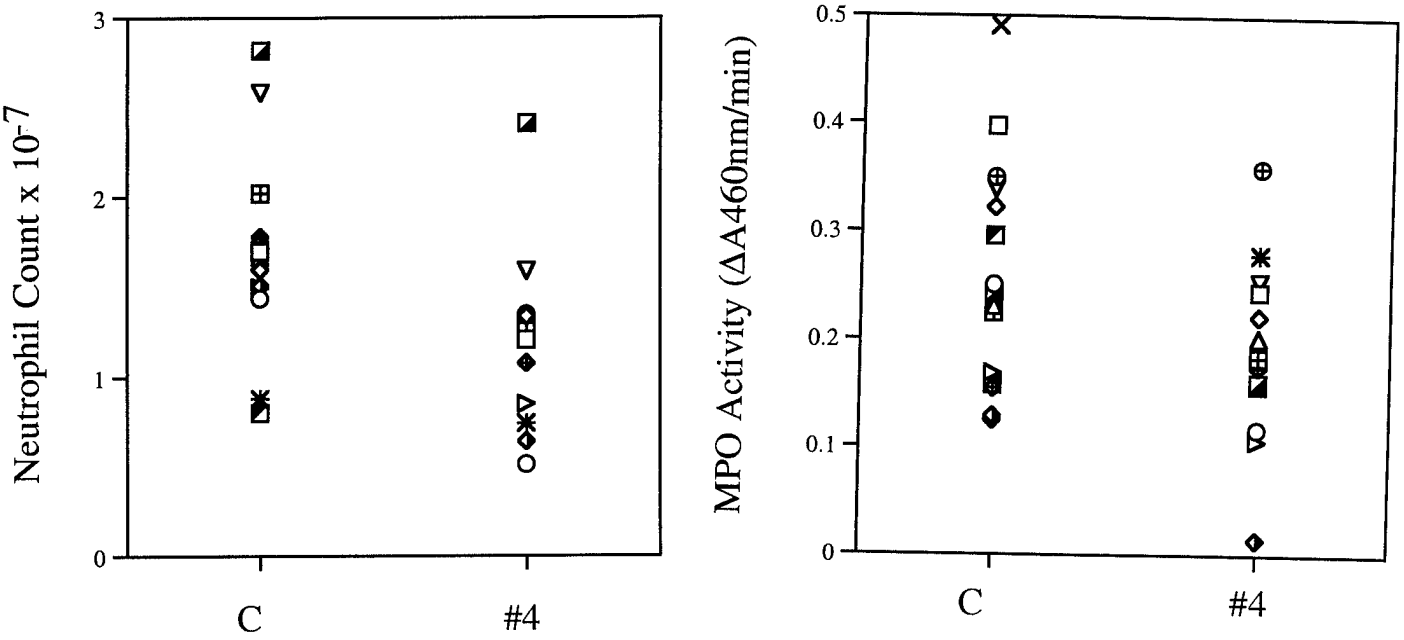

FIG. 2. Mapping of the amino acid residues within the sequence DLWDWVVGKPAG that are critical for MAb NS19-9 binding. The peptide sequence was scanned by substitution of each amino acid with other L-amino acids by spot synthesis and resulted peptides were tested for MAb NS19-9 binding or varying number of amino acids was truncated. The number of peptides is 163 (6 rows, 27 spots each). First spot represents lead peptide with amino acid sequence DLWDWVVGKPAG. (A) Control membrane after blotting with BSA instead of specific NS19-9 MAb and secondary antibody; (B), membrane after immunoblotting with NS19-9 MAb and the secondary antibody.

Most substitutions of residues 2 to 5 abolished NS19-9 binding, with Trp3 and Trp5 being the most critical. Identical sets of amino acids (His, Tyr, Ala, Asp, Glu, Lys, Arg, Ser) were shown to completely abolish antibody binding, while Met significantly decreased the signal upon replacement of either of these residues (Trp 3 and 5). Similarly, substitutions of Leu2 with Ala, Asp, Tyr, Glu, Lys, Arg, and Ser as well as substitution of Asp4 with Glu, Ser, Pro, Val, Met, and Tyr completely abolished MAb binding. All peptides generated by truncation of amino acids 2 to 8 from the N-terminal from the peptide were no longer recognized by the antibody. Most substitutions upstream from position 5 (positions 6-9) allowed for MAb binding with no evident preference for substituted amino acids. Although, substitution analysis failed to identify significant differences in binding as a result of substitution of residues 9-12 within the C-terminal half of the peptide, MAb did not detect truncated peptides within this region implying the importance of the C-terminus for MAb binding.

Comparison of signal intensities on the peptide array revealed that some substitutions led to enhanced NS19-9 binding allowing for identification of several peptides with increased binding affinity to the antibody. Improvement of peptide binding was achieved mainly by substitution of residues 5 to 12 within the lead peptide, whereas no amino acid exchange at N-terminus (residues 1-4) led to the increased binding. The replacement of residues with amino acids containing polar groups such as Glu and Asp clearly showed an enhancing effect at the C-terminus but not the N-

terminus. Array analysis failed to reveal significant differences in the binding intensities between the peptides substituted at different positions, suggesting that single substitution at any position in this region with carboxyl groups can enhance the interaction with the MAb binding site. In addition, substitutions with Ile, Ala and Ser also improved MAb binding. Similarly, the simultaneous replacement of several residues with clusters of amino acids upstream from position 6 demonstrated enhanced binding. The highest intensity signal was, however, observed with peptide DLWDFVVGKPAG containing a single substitution at position 5 with Phe (Appendix 2).

In summary, a distinct pattern of substitutions that led to increased or abolished signal intensities with respect to the C- and N-terminus suggests that the region close to the N-terminus might contribute to the specificity of the interaction with NS19-9. Amino acids close to the C-terminus appear to add significantly to the affinity of ligand binding.

Structural analysis of peptide mimics of SA-Lea. The lead peptide DLWDWVVGKPAG (#4) and the array selected peptide DLWDFVVGKPAG (#44) were synthesized individually and their binding specificities to NS19-9 MAb was assayed by competitive solid phase enzyme linked immunosorbent assay (Appendix 2). Both peptides inhibited binding of MAb NS19-9 to a solid phase adsorbed cognate carbohydrate antigen SA-Lea in a dose dependent manner. IC_{50} of the substituted peptide DLWDFVVGKPAG was established at 70 μM whereas the IC_{50} of the lead peptide DLWDWVVGKPAG was 700 μM. This 10-fold lower IC_{50} value for the array-selected peptide reflects a higher binding affinity of this peptide as compared to the lead peptide. These data suggest the DLWDFVVGKPAG peptide displays a better fit into MAb binding site as compared with the original peptide. No significant binding of MAb NS19-9 was observed with unrelated peptide in the concentration range up to 5 mM.

Secondary structure prediction using circular dichroism spectra and based on a neutral net algorithm indicated some propensity of both peptides #4, DLWDWVVGKPAG and DLWDFVVGKPAG, #44 to assume extended or helical structures centered at the mid-chain W/FVVG domain (Appendix 3). Both peptides DLWDWVVGKPAG and DLWDFVVGKPAG highlight the functional role played by the aromatic-X-aromatic motif within the peptide. It is possible that these structure types are realized within the antibody-combining site (16). Turn conformations appear to play an important role in E selectin recognition based on structure activity relations of modified Ser-Glu dipeptides that bind to E selectin. The increased binding of peptide with substitution of Phe for Trp would suggest that the Phe directly contributes to MAb and that hydrophobic stacking interactions are important for increased antibody binding and consequently antigenic mimicry. This assertion is supported by X ray crystallographic and molecular modeling studies of carbohydrate mimicking peptides.

Experimental Metastatic Model. SA-Lea appears to mediate the adhesion of many carcinoma tumors to human umbilical vascular endothelial cells in multiple *in vitro* studies. To establish an *in vivo* experimental metastatic model and to investigate SA-Lea supported adhesion of tumor cells to lung endothelium, B16F10 murine melanoma cells (syngeneic with C57Bl/6 haplotype) were stably transfected with α1-3/4-fucosyltransferase cDNA (17) in order to express SA-Lea antigen on the tumor cell surface. The B16F10 clone of B16 cell line does not express E-selectin ligands SA-LeX or SA-Lea as demonstrated by FACS analysis (not shown). The tumorigenic dose for the C57Bl/6 syngeneic tumor cells was established by i.v. injection of various numbers of cells. A 1×10^5 dose was chosen for further experiments as countable lung metastases were observed after i.v. injection of 1×10^5 of B16F10FTIII cells expressing SA-Lea after 21 days (Appendix 2).

The role of tumor cell adhesion to vascular EC via E-selectin and its ligand SA-Lea interaction in metastasis formation was established *in vivo* in two ways. First, to directly assess the role of E-selectin in tumor colonization *in vivo*, we determined the ability of B16F10 murine melanoma cells expressing SA-Lea to colonize in the lung of E-selectin knock out (KO) mice in

parallel wild-type C57Bl/6 mice (Fig. 3). Mice of both strains received i.v. injection of 1 x 10^5 B16F10FtIII tumor cells and examined 3 weeks later. Only 20% of E-selectin deficient animals injected with tumor cells developed small numbers of lung metastasis, while the rest of the E-selectin KO mice showed no detectable lung tumor nodules. Statistical analysis gave P values < 0.009 for E-selectin KO as compared to the control group (Fig. 3 *A* and *C*), respectively. Small nodules were observed in a few E-selectin KO mice that developed tumors whereas all animals in the control group developed multiple metastasis and some of them died earlier than 3 weeks. The results demonstrate that lung metastasis of tumor expressing SA-Lea antigen is completely abrogated in most of the genetically manipulated mice that lack expression of E-selectin, highlighting the critical role of E-selectin in mediating carcinoma metastasis *in vivo*.

To further test the hypothesis that SA-Lea expression supports adhesion of tumor cells to E-selectin on EC, we tested the inhibitory effect of peptide mimicking SA-Lea antigenic structure DLWDFVVGKPAG on lung colonization of B16F10FTIII cells. One x 10^5 tumor cells expressing SA-Lea were admixed with a solution containing 1 mg of the peptide DLWDFVVGKPAG, followed by administration of the mixture to groups of mice. Animals were euthanized after 21 days following tumor challenge and the number of metastasis was enumerated in each. Administration of the peptide DLWDFVVGKPAG abrogated on average 50% lung colonization of the B16F10FTIII induced tumor nodules developed in control animals; some mice being completely devoid of tumor nodules (Fig. 3*B*). The difference was highly statistically significant ($p<0.008$). In addition, B16F10FtIII cells in C57Bl/6 mice developed large tumor masses with diffused infiltration of tumor cells and some mice died before the termination of the experiment (median 16 days). This finding strongly suggests that the interaction of SA-Lea carbohydrate tumor-associated antigen with E-selectin expressed on vascular EC is an important step in establishing tumor metastasis.

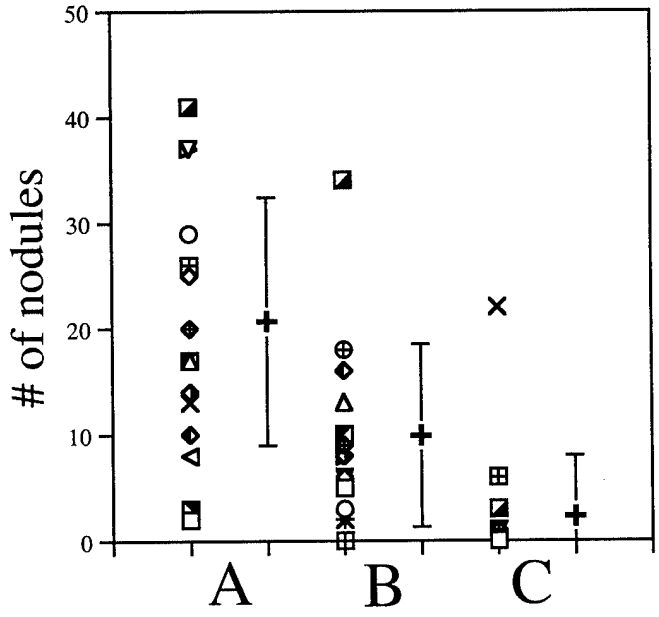

FIG. 3. Inhibition of lung experimental metastases with peptide DLWDFVVGKPAG. Tumor cells were admixed with the specific or unrelated peptide solution (1 mg per mouse) and animals were inoculated with 1 x 10^5 B16F10FtIII tumor cells in 200 µl volume of PBS via tail vein. Results are from 4 experiments (5 mice in each group) are shown. Each dot represents enumerated tumor nodules in one lung in experimental group of C57Bl/6 mice treated with the peptide (panel B), control group of C57Bl/6 mice treated with unrelated peptide (panel A) and E-selectin KO mice of C57Bl/6 background (panel C). Statistical analysis using a nonparametric unpaired t test gave a two-tailed P values <0.008 and 0.009 for animals treated with peptide and E-selectin KO, respectively, as compared to control group. The horizontal bars represent median values and vertical bars denote standard deviation.

Discussion

To study the role of carbohydrate tumor-associated determinants in metastasis, we hypothesized that structurally equivalent compounds with an ability to interfere with an interaction of carbohydrates with endogenous lectins will be able to interrupt the metastatic process. Such a compound should also prove useful in defining the importance, the mechanism, and the stage of carbohydrate involvement in this process.

We have previously determined that peptide mimicking SA-Lea has an ability to inhibit neutrophil recruitment into the peritoneal cavity in experimental acute inflammation. Since both neutrophils and carcinoma cells express ligands for E-selectin, we hypothesized that early steps of metastatic colonization may be considered equivalent to the initiation of the inflammatory process.

Tumor colonization appears to be highly E-selectin dependent, as the incidence of metastasis was completely abrogated in E-selectin KO mice (18) in our study. The complete inability of tumor cells colonization in the lungs of E-selectin KO animals may result from the lack of initial stages of adhesion to lung endothelium required for tumor cell migration into the subendothelial space as well as impaired angiogenesis in which E-selectin is involved (19) resulting in reduced number of nodules but not tumor growth after the micrometastases are established. The role of E-selectin was suggested in previous studies where metastases formation *in vivo* was completely or partially abrogated as a result of treatment with E-selectin or E-selectin specific MAb (3,4).

In our study, the identification of peptides mimicking E-selectin ligand, SA-Lea, inhibiting metastasis of tumor cells expressing this structure provides an evidence that SA-Lea is an important determinant in the process of metastasis formation. The 50% inhibition of tumor metastasis achieved upon administration of the peptide antagonist of SA-Lea expressed on the B16F10 tumor cell surface in our model can be explained by interruption of the initial steps of cascade of inhibitory events initiated by tumor cell adhesion with this conformational equivalent of the SA-Lea structure. These findings suggest that expression of SA-Lea leads to tumor specific colonization *in vivo*, highly likely via interaction with E-selectin. Tumor cells expressing SA-Lea have been reported to mediate tumor colonization specifically in organs with high expression of E-selectin in E-selectin transgenic mice (5). Although the reduced number of metastases was the prevalent effect of peptide treatment as compared with the control group, large lung tumor masses were observed in untreated animals. The experimental evidence suggests that E-selectin adhesion molecule and carbohydrate structures, SA-Lea and SA-LeX, are associated with capillary tube formation and neovascularization necessary to maintain metastasis (20). This might indicate that the anti-angiogenic mechanism of tumor growth inhibition in peptide treated animals also takes place.

7. Key research accomplishments:

a. Establishing critical role of E-selectin in vivo in mediating metastases of tumor cell expressing SA-Lea determinant

b. establishing in vivo role for SA-Lea in tumor colonization

c. proving that peptides mimicking carbohydrate structures can be developed and their structural properties with respect to the protein binding can be improved by amino acid substitutions

c. proving that such a structural mimics of carbohydrate are useful in studying the processed in which these ligands are involved, such as inflammation and metastasis

d. proving that these compounds may be useful in developing reagents to treat these pathologic conditions

8. Manuscripts and funding

1. O, I., Otvos, L., Jr., Kieber-Emmons, T., and Blaszczyk-Thurin, M. Peptides mimicking sialyl-Lewis A isolated from a random peptide library and peptide array. Annals New York Academy of Sciences, 1999, in press.

2. O, I., Otvos, L., Jr., Kieber-Emmons, T., and Blaszczyk-Thurin, M. Inhibition of metastasis by a peptidomimetic of sialyl-Lewis a. Submitted.

The results obtained from the research supported by this award are the basis of the RO1 application to NIH, July 1st deadline.

9. Conclusions

Our study for the first time determined an *in vivo* importance of SA-Lea carbohydrate tumor-associated antigen in metastasis using a murine tumor model established by us. Peptides mimicking carbohydrate determinant were effective in inhibiting metastasis of tumor cells expressing this structure *in vivo*, demonstrating that it interferes with critical events for metastases formation. The identified peptides were also effective in reducing inflammation *in vivo* highly likely via the same mechanism, i.e. interruption of carbohydrate ligands expressed on neutrophils and for E-selectin of EC. E selectin is an postulated adhesion molecule which recognizes SA-Lea, and we demonstrated that tumor growth in E-selectin KO mice was completely abrogated. These data provide direct evidence that SA-Lea and E-selectin are important in inflammation and metastasis formation *in vivo*. These studies provide new information on selectin-carbohydrate/ligand interactions in a metastatic cascade, and may help in the design of improved therapeutic intervention in malignancies and other diseases involving these interactions.

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11. Appendicies

1. O, I., Otvos, L., Jr., Kieber-Emmons, T., and Blaszczyk-Thurin, M. Peptides mimicking sialyl-Lewis A isolated from a random peptide library and peptide array. Annals New York Academy of Sciences, 1999, in press.

2. O, I., Otvos, L., Jr., Kieber-Emmons, T., and Blaszczyk-Thurin, M. Inhibition of metastasis by a peptidomimetic of sialyl-Lewis a. Submitted.

13. Publications:

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2. O, I., Otvos, L., Jr., Kieber-Emmons, T., and Blaszczyk-Thurin, M. Inhibition of metastasis by a peptidomimetic of sialyl-Lewis a. Submitted.

Personnel involved:

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Insug O, Ph.D., Investigator

Peptides Mimicking Sialyl-Lewis A Isolated from a Random Peptide Library and Peptide Array

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Abbreviations: MAb, monoclonal antibody; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SA, Sialyl; Le^a/X, Lewis $^a/X$; ELISA, enzyme linked immunosorbent assay

Cell surface carbohydrate structures are an important class of tumor antigens. SA-Le^a and its structural isomer SA-LeX have been identified as carbohydrate structures expressed on many carcinomas (1) and recently both structures have been shown to represent functional ligands of selectins. The crucial role of selectin-dependent neutrophil adhesion in their recruitment process and metastasis was confirmed by in vivo blockage of E-selectin-dependent interaction (2). These data imply that similar molecular mechanisms do indeed underlie inflammation and metastasis, and that similar therapeutic approaches can be used to intervene both processes. Highly diverse peptide libraries offer many distinct advantages over difficult chemical or enzymatic synthesis of complex carbohydrates, providing a notably inexpensive and rapid identification and optimization of novel ligands. The peptides described here provide excellent leads for development of potent carbohydrate -protein interaction and in particular anti-metastatic and anti-inflammatory therapeutic agents.

Materials and Methods.

MAb NS19-9 was generated at The Wistar Institute (3). Peptides were synthesized at Research Genetics, Inc. SA-Le^a was obtained from Glycotech, Inc. (Rockville, MD).

The 12-mer peptide library for these studies was obtained from Invitrogen Inc. (Carlsbad, CA)(4). The ability of peptides to block MAb recognition of SA-Le^a carbohydrate was determined in competition ELISA using various peptide concentrations. IC_{50} values were calculated by non-linear least-squares regression of a four-parameter logistic equation. An array of 163 synthetic 12-mer peptides was synthesized by standard F-moc chemistry on polyethylene glycol modified cellulose membrane at the Wistar Institute and tested for binding of MAb NS19-9 (5).

Results.

Random peptide library screening. Several bacterial clones that bind the SA-Le^a specific NS19-9 MAb were isolated and sequenced. Clones isolated with the carbohydrate-specific MAb in the final selection cycle were tested for protein expression using SDS-PAGE and identified after probing by Western blot with the MAb. Peptide library screening yielded families of peptides with unique consensus sequences. Two distinct consensus sequences GXWXXVLEG and VVGXP were identified in families of peptides isolated with MAb NS19-9 (Table 1). This may indicate that peptides based on two different motifs isolated with the same MAb can mimic different structural topographies of SA-Le^a carbohydrate and these subsets of peptides may very likely represent non-overlapping surfaces of cognate antigen.

Identification of sequences critical for MAb binding using peptide array. To identify the amino acid residues critical for MAb binding and to analyze amino acid substitutions which might improve peptide-MAb interaction, we generated an array of 163 peptides based on the amino acid sequence of peptide #4 isolated from family II with MAb NS19-9 and measured binding of MAb (Fig.1). Results suggest that most substitutions at the very N-terminus (residues 1 and 2) were well

tolerated and did not influence MAb binding, whereas critical residues were clearly identified at positions 3 to 5. The most critical single amino acid for MAb binding was Trp at position 3 since most of the amino acid substitutions at that position abolished binding. Substitutions at this position with H, Y, A, D and S completely abolish binding whereas M significantly decreased the MAb binding. Similarly, substitutions of W at position 5 with H, A, R and K completely abolished MAb binding. Several of the substitutions within the identified concensus sequence, in particular VVGK, completely abolished MAb binding whereas others allowed for MAb binding. No preference for amino acids was evident from the substituted peptides within positions 10 to 12 of the C-terminus. In contrast, several amino acid substitutions resulted in increased binding of the peptides for MAb. The most favorable amino acid with respect to MAb binding was substitution of Trp at position 5 with Phe resulting in the sequence DLWDFVVGKPAG that displayed increased binding affinity as compared to the original peptide. Amino acid substitutions within the consensus sequence, at positions 6 to 10 mainly with K, R, and E also resulted in higher binding of MAb as compared with the original peptide. These data demonstrate that peptides with higher binding properties for the anti-carbohydrate MAb were selected using the peptide array approach. These results further confirm that aromatic amino acids may play an important role in mimicking carbohydrate structures.

Synthetic peptides mimic the carbohydrate antigen detected with MAb NS 19-9. To test whether peptide #4 and peptide A#44 are true mimics of SA-Le^a , competition ELISA was carried out in order to determine the concentration of peptides required for blocking of 50% of MAb binding to native carbohydrate antigen. Both peptides blocked the binding of NS19-9 to a fixed amount of carbohydrate antigen in a dose dependent manner. The IC_{50} for peptide #4 blocking of MAb-SA-Le^a binding was 900 μM. Peptide A#44 showed more prominent inhibition of the MAb-SA-Le^a binding as compared to the peptide #4 as demonstrated with the IC_{50} value of 100 μM. This suggests that the peptides sterically interfere with MAb binding to carbohydrate antigen, implying that the sequence, DLWDWVVGKPAG and DLWDFVVGKPAG represent solvent-accessible epitopes and that the peptides represent cognate determinants for the antibody. This also suggests that substitution of Phe within the original amino acid sequence generated a peptide with higher affinity for the MAb mimicking topography for SA-Le^a.

Secondary structure of peptides mimicking carbohydrate. Both peptides #4 and A#44 highlight the functional role played by the aromatic-X-aromatic motif within the peptide. Secondary structure analysis indicates some propensity for extended and helix structures centered on the W/FVVG region using a neutral net analysis. It is possible that these structure types might be realized within the antibody-combining site. This is consistent with modeling and crystal analysis of this motif type suggested to adopt type I and type II turns within the antibody-combining site. The increased

binding of peptide with substitution of Phe for Trp would suggest that the hydrophobic stacking interactions are important for increased antibody binding and consequently antigenic mimicry.

Comments.

Functional equivalence of chemically dissimilar molecules such as carbohydrates and proteins sharing common surface topology has been identified previously as a naturally occurring phenomenon. Combinatorial technologies available in recent years have provided an avenue to dissect the molecular basis for such mimicry. Using a combinatorial library screening approach we isolated families of mimics of tumor associated antigen and E-selectin ligand. We have chosen one sequence as a lead peptide to delineate the specific residues that may contribute to the mimicry of carbohydrate structures by re-screening of a peptide array. The MAb could tolerate a variety of amino acid substitutions within the lead peptide sequence, still retaining functional specificity. Furthermore, cross-reactive peptides of higher affinity were identified. This suggests that different amino acids can improve structural mimicry within identified peptide. Since families of peptides with different consensus sequences were identified with the same MAb further suggests that indeed different residues act as structural mimics. Alternatively, different consensus sequences mimic different topographies of a carbohydrate epitope recognized by the antibody. The prospect of finding mimicking peptides of carbohydrate tumor antigens that competitively inhibit carbohydrate-specific receptors will allow for the design of antagonists of E-selectin and other endolectins with enhanced therapeutic potential to prevent metastasis.

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Table 1. Peptide sequence families mimicking SA-Lea carbohydrate structure.

I	#2:	VGIWSVVSEGSR	II	#1:	RCSVGVPFTMES
	#3:	QDGVWEHVLEGG		#4:	DLWDWVVGKPAG
	#15:	VELSGRGGLCTW		#12:	VIGAASHDEDVD
	#18:	TIEPVLAEMFMG		#14:	DKETFELGLFDR
				#15:	FSGVRGVYESRT
				#19:	PDDAPMHSTRVE

Figure 1. (A) Reactivity of MAb NS19-9 with a series of solid-phase single amino acid substituted dodecapeptides based on the peptide #4. (B) Background binding to the membrane, without primary antibody.

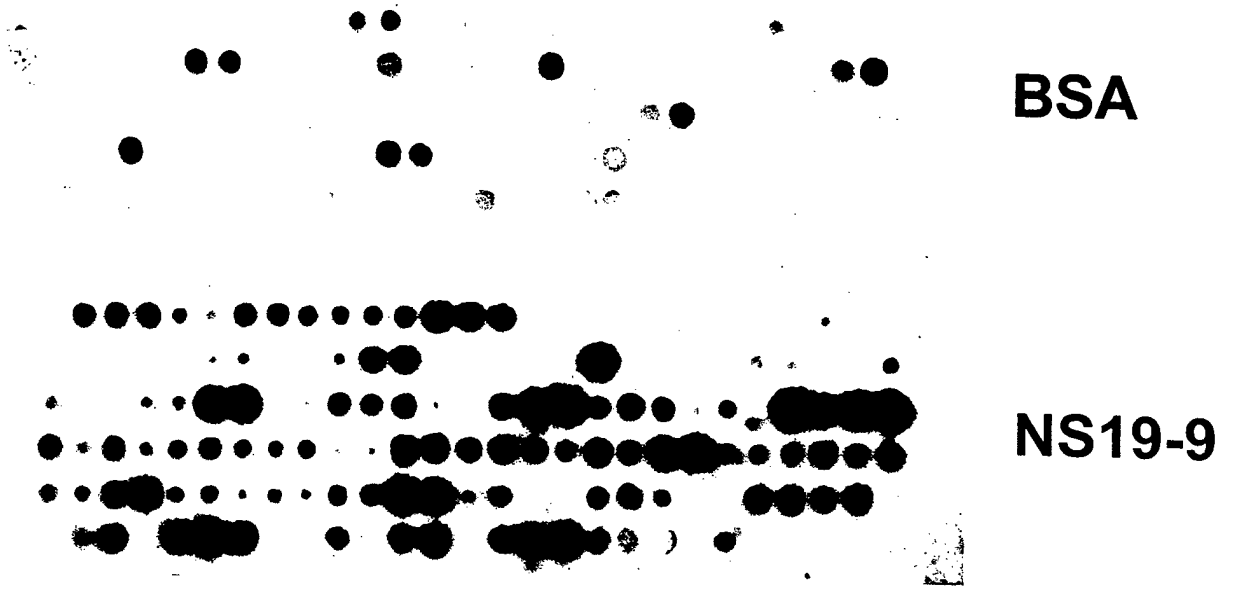

Figure 1

Inhibition of Metastasis by a Peptidomimetic of Sialyl-Lewis a*

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[1] The abbreviations used are: SA-Le[a], sialyl Lewis a; SA-LeX, sialyl Lewis X; KO, knock out; MAb, monoclonal antibody; IC_{50}, 50% inhibitory concentration; EC, endothelial cells; DMF, dimethylformamide; PBS, phosphate-buffered saline; CD, circular dichroism; i.v., intravenous.

Sialyl-Lewis a (SA-Le^a) and its structural isomer Sialyl-Lewis X (SA-LeX) are expressed on many carcinoma cells and are E-selectin ligands. Here, we demonstrate a critical role of E-selectin in mediating metastasis *in vivo* by completely abolishing metastasis formation of SA-Le^a expressing B16 tumor cells in E-selectin knock out (KO) mice. Furthermore, we demonstrate that a peptide mimic of SA-Le^a mediates a 50% inhibition of lung colonization of tumor cells in an *in vivo* murine tumor model. A SA-Le^a mimicking peptide with the sequence DLWDWVVGKPAG was previously identified by screening a combinatorial peptide library using a SA-Le^a specific monoclonal antibody (MAb) NS19-9. In the present study, peptide array composed of peptides with substituted residues within the DLWDWVVGKPAG sequence maps key residues interacting with the NS19-9 binding site. Amino acid substitution analysis reveals several specific residue replacements that led to increased NS19-9 binding. The replacement of Trp 5 with Phe, results in a change of the secondary structure of the peptide and 10-fold lower 50% inhibitory concentration (IC_{50}) for MAb binding as compared to the lead peptide. The study provides for further elucidation of the fine specificity of oligosaccharide-protein interactions and the role played by carbohydrate tumor-associated antigens in the metastatic process.

Tumor metastasis is a multistep process requiring detachment of malignant cells from the primary tumor, penetration of blood or lymph vessels and attachment to endothelium of distant organs and formation of new tumor (1). Endothelial adhesion molecules of the selectin family recognize carbohydrate antigens expressed on the tumor surface and mediate the initial interaction between tumor cells and endothelium. E-selectin is a tumor necrosis factor α (TNF-α)[1] and interleukin 1β (IL-1β inducible, calcium-dependent molecule, that is expressed on vascular endothelium during the process of leukocyte recruitment (2). E-selectin binds to glycoconjugates carrying the terminal tetrasaccharide sialyl-LeX (SA-LeX), [NeuAcα2,3Galαlβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ1-R] (3), but displays higher affinity for the SA-Le^a structure ([NeuAcα2,3Galβ1,3(Fucα1,4) GlcNAcβ1,3Galβ1,4Glcβ1-R], a positional isomer of SA-LeX (4). Metastatic potential is correlated with expression of these structures (5, 6). In particular, E-selectin is postulated to be the most efficient selectin, mediating the rolling of cancer cells to endothelial cells (EC) (7-12). Although adhesion pathways utilized by different tumors show considerable diversity, recent reports (11, 12) suggest that at least one member of the selectin family of molecules and SA-Le^a and/or SA-LeX carbohydrate ligands, might be involved in tumor metastasis, mediating binding of blood-borne tumor cells to vascular endothelium. Cancer cells that express both SA-Le^a and SA-LeX undergo SA-Le^a-mediated adhesion almost exclusively, possibly due to the higher affinity for the SA-Le^a structure, or differential presentation of this

oligosaccharide determinant. Thus, SA-Le^a might play a major role as a ligand in the E-selectin dependent adhesion to EC *in vivo* (13-15).

The expression of ligands for E-selectin by both neutrophils and cancer cells raises the possibility that the basic mechanisms that underlie initial step of metastasis formation are equivalent to those mediating the rolling step in inflammatory process; e.g., through interaction of endothelial selectins with the tumor-associated carbohydrate ligands SA-LeX and SA-Le^a. The crucial role of selectin-dependent neutrophil adhesion and carcinoma cell-adhesion implies that *in vivo* blockage of selectin-dependent interactions can decrease leukocyte mobilization and the incidence of metastases. Controlling of the selectin-dependent interaction has been considered as an anti-inflammatory approach by many laboratories (16-23). Although there is no direct evidence of involvement of E-selectin in naturally occurring tumor metastases in humans, the administration of E-selectin-specific antibody and soluble E-selectin abrogates the formation of hepatic metastasis and lung colony formation *in vivo* in experimental animals (24-26). Furthermore, tumor expressing SA-Le^a metastasize specifically to the organs of increased levels of E-selectin in E-selectin transgeneic mice, whereas no metastasis were observed in wild-type mice (27). Further support for this association comes from *in vitro* observations that SA-Le^a and SA-LeX mediate adhesion of tumor cells with E-selectin and human umbilical vascular endothelial cells (7-15). These data imply that similar molecular mechanisms do indeed underlie early step in inflammation, i.e., leukocyte rolling and metastasis, i.e., adhesion of tumor cells to

EC and that similar therapeutic approaches can be used to intervene with these processes.

Although E-selectin ligands are expected to inhibit tumor formation, SA-Le^a and SA-LeX are not easily prepared in large quantities. Developing reagents that are sterochemically equivalent to carbohydrate ligands for E-selectin that can effectively block tumor colonization *in vivo* might provide an effective treatment of the metastatic process. The role of carbohydrate ligands in mediating adhesion indicates the potential for carbohydrate "structure-based" or mimetic pharmacological agents that compete with Lewis antigens as "anti-adhesion" therapeutics. Many peptide mimics of carbohydrate structures have been described in the literature (28-39) including those binding with high affinity to E-selectin (40). E-selectin mimics tested so far have really only focused on mechanisms associated with the idea of lymphocyte rolling and not on metastasis. In contrast, a peptide that mimics the GD1 ganglioside, also involved in cell adhesion and metastasis of melanoma cells, has been recently described (41). This peptide isolated from a peptide phage display library using an anti-GD1 antibody inhibits metastasis in an *in vivo* model.

Here we demonstrate that SA-Le^a and E-selectin interaction participate in tumor metastatic dissemination. Mice that lack expression of E-selectin [E-selectin knock out (KO)] are shown to be completely resistant to tumor metastasis. Furthermore, administration of a peptide mimic of SA-Le^a is able to effectively modulate tumor cell dissemination. The results demonstrate the important role for both E-selectin and SA-Le^a in promoting the tumor metastatic process and that

peptides mimicking SA-Le^a presumably act as antagonists for E-selectin, interfering with E-selectin function *in vivo*. Understanding the mechanism of carbohydrate recognition my lead to the development of new concepts and new strategies to develop antagonists for this biologically important interaction.

EXPERIMENTAL PROCEDURES

Antibodies and Peptides–MAb NS19-9 was generated at the Wistar Institute and its specificity was previously characterized (42). Peptides were synthesized by standard solid-phase strategies and HPLC-purified at the Peptide Synthesis Facility of The Wistar Institute or by Research Genetics (Huntsville, AL). The structures were confirmed by fast-atom bombardment mass spectrometry at The Wistar Institute Protein Sequencing Facility. Synthetic multivalent SA-Le^a-polyacrylamide matrix conjugate was purchased from Glycotech, Inc. (Rockville, MD).

Random Peptide Library and Library Screening–A dodecapeptide (FLITRX) library (Invitrogen, Carlsbad, CA) was screened using the SA-Le^a specific NS19-9 MAb as described (43). Briefly, an aliquot containing at least 2 X 10^{10} cells to ensure full representation of peptides, was grown to saturation for 15 h in IMC/amp100 medium (M9 medium containing 1 mM $MgCl_2$ supplemented with 0.5% glucose, 0.2% casamino acids and 100 μg/ml ampicillin). The expression of thioredoxin with incorporated 12-mer peptide sequences was induced with tryptophan by incubation for 6 h. The induced bacteria were panned on a MAb-coated tissue culture plate (20 μg/ml). Bound bacteria were washed gently and eluted cells are collected by rinsing the plate with fresh IMC/amp100 medium collected eluted cells. The entire selection process was repeated four more times. Colonies of isolated bacteria were grown on ampicillin-containing plates. Individual colonies were isolated and

grown as a small scale culture (2 ml) in a IMC/amp100 medium with tryptophan for 6 h and analyzed using Western blot (44).

Peptide Array–A peptide array of 163 unique peptides was generated by substituting all amino acids for each individual amino acid in a lead peptide (DLWDWVVGKPAG; #4) identified by combinatorial library panning with MAb NS19-9. An array of synthetic 12-mer peptides was synthesized using 90 x 130 mm polyethylene glycol-modified cellulose membrane functionalized with approximately 4 nmole/mm^2 amino groups, manufactured by Abimed (Lagenfeld, Germany). Standard Fmoc chemistry was used throughout (46), according to Abimed's instructions (47). The protected and activated amino acids were spotted using an Abimed ASP 422 robotic arm. All washing, dyeing and deprotection steps were done manually. The activated C-terminal amino acids were spotted leaving 10 mm space in each direction, at the concentration of 0.5 M in N-methyl pyrrolidone. A volume of 0.5 ml provides spot of 7-8 mm in diameter. Activation of the amino acids with dicyclohexyl-carbodiimide and N-hydroxy-benzotriazole was done 30 min before spotting. After each coupling cycle, the paper was washed with 12% acetic anhydride dissolved in N,N'-dimethylformamide (DMF) twice for a total of 10 min to endcap all unreacted amino groups. Repetitive removal of the Fmoc groups was achieved by two treatments with 20% piperidine in DMF for 5 and 10 min, respectively. The second and consecutive amino acids were coupled in a 1.1 molar excess, and were spotted 3-4 times depending upon the outcome of the bromophenol blue assay of the couplings. After the coupling and deprotection steps the membrane was washed thoroughly with DMF and ethanol, dried and stained

with bromophenol blue dissolved in DMF. After successful coupling the paper remains colorless; after successful deprotection steps the peptide dots turn deep blue. The coupling steps were repeated until all peptide spots remain colorless. The N-terminal amino acids at the end of the syntheses remained uncapped. Final removal of the side-chain protecting groups was performed by washing the paper with mixture of 12.7 ml trifluoracetic acid, 3.7 ml *m*-cresol, 3.7 ml thioanisole, 3.7 ml water and 3.7 ml ethanedithiol. After cleavage, the paper was washed several times with ethyl alcohol, DMF, water and methyl alcohol, and dried. The peptide array library was tested for binding of MAb NS19-9. The cellulose filter was blocked with 5% non-fat milk in phosphate buffered saline (PBS) for 1 h at room temperature followed by washing with PBS. Filters were incubated with goat anti-mouse immunoglobulin G conjugated with horse radish peroxidase (1 μg/ml) for 1 h. Filters were washed five times in PBS-T (0.05% Tween in PBS, v/v) and developed using chemiluminescence reagent followed by autoradiography as described in Western blot.

Peptide Inhibition Assay–For peptide competition assays, test peptides at concentrations ranging from 10 nM to 1 mM were preincubated with 100 μl of the MAb NS19-9 (5 μg/ml) diluted in 10% μ-globulin free horse serum/PBS at room temperature. Fifty μl of preincubated inhibition complex antibody-peptide is added to each well of 96-well plate and incubated at 30-37 °C for 1 h. After 1 h incubation, MAb/peptide complex mixtures were transferred to wells precoated with a constant amount of neoglycoprotein containing coupled multivalent carbohydrate

determinant (SA-Le^a-polyacrylamide matrix) (5 μg/well) and allowed to bind for 1 h followed by blocking with 10% γ-globulin-free horse serum for 2 h at room temperature. Wells were washed with 100 μl PBS four times. Goat anti-mouse immunoglobulin G conjugated to horse radish peroxidase (Boehringer-Manheim, Indianapolis, IN) was diluted 1000-fold with 10% γ-globulin free horse serum/PBS and 100 μl was added in each well and incubated at 30-37 °C for 1 h and washed with 100 μl PBS five times. Ten mg substrate for horse radish peroxidase, tetramethylbenzidine dichloride (TMB) (Sigma Chemical Co., St. Louis, MO) was dissolved in 10 ml of 0.05 M phosphate-citrate buffer, pH 5.0, containing 0.03% sodium perborate (Sigma Chemical Co.). One-Hundred μl substrate was added in each well and incubated at room temperature for 10 min. The developed blue color was read at 450 nm after stopping the reaction with 100 μl 1 M phosphoric acid. Fifty % inhibitory concentration (IC_{50}) were calculated by non-linear least-squares regression to a four-parameter logistic equation.

Circular Dichroism (CD)–CD spectra were taken on a Jasco J720 instrument at room temperature in a 0.2 mm pathlength cell. Double distilled water and spectroscopy grade trifluoroethanol were used as solvents. The peptide concentration was 0.51 mg/ml, determined by quantitative reversed-phase HPLC (48). The algorithms provided by JASCO accomplished curve smoothing. Mean residue ellipticity is expressed in degrees cm_2/dmole by using a mean residue weight of 110. Because the secondary structures of the peptides by the current computer-

assisted curve analyzing algorithms show a high error rate, the CD spectra evaluations were based on comparison with known peptide conformations (49).

B16F10 Murine Melanoma Cells Expressing SA-Le^a Structure–The murine B16F10 melanoma cell line was obtained from the American Type Tissue Collection (Rockville, MD). cDNA encoding α1-3/4 fucosyltransferase (FTIII) (50) was digested by *Hind*III and *Not*I restriction enzymes from the πH3M vector containing FTIII cDNA provided by Brian Seed, Massachusetts General Hospital (Boston, MA), and cloned into pCDNA3(*neo*) vector, which resulted in pCDNA-FTIII. B16F10 cells were transfected with pCDNA3 vector containing cDNA encoding α1-3/4 fucosyltransferase pCDNA-FTIII using Effectene (Qiagen, Chatsworth, CA) as recommended by the manufacturer. The resulting cell line, B16F10FTIII, expresses SA-Le^a carbohydrate structure as demonstrated by flow cytometry analysis using MAb NS19-9 as compared to the parental B16F10, which did not show staining with this antibody. The transfected cells were grown in the presence of G418 (500 μg/ml) (Gibco-BRL, Grand Island, NY) for 10 days. To ensure the homogeneity of the transfected cells with respect to the expression of SA-Le^a, the cells were subjected to cell sorting using SA-Le^a specific MAb NS19-9 followed by FITC-conjugated goat anti-mouse immunoglobulin. Analysis and sorting were carried at The Flow Cytometry Facility at The Wistar Institute.

Experimental Metastatic Model–Six- to 8-week-old C57Bl/6 female mice were purchased from Jackson Laboratory (Bar Harbor, ME). E-selectin KO mice of C57Bl/6

background (51), lacking E-selectin expression, were kindly provided by Dr. Daniel Bullard (University of Alabama at Birmingham, AL), and bred at The Wistar Institute's Animal Facility. B16F10FTIII cells positive for SA-Le^a were grown *in vitro* in Iscove's culture medium supplemented with 10% FBS for 1 week before injecting into mice. Cells were collected and washed twice in Iscove's medium without serum and suspended in PBS. One X 10^5 tumor cells in a volume of 200 µl in PBS were inoculated by intravenous (i.v.) route via tail vein. To test the effect of the peptide, animals were inoculated with a single dose of peptide at the time of tumor challenge. One mg of peptide was admixed with the tumor cells and together injected via i.v. route. Control animals received injection of tumor cells admixed with the same amount of unrelated peptide. Mice were euthanized after 3 weeks following tumor cells injection and lung and other organs were examined under dissecting microscope for the presence of tumor nodules. The lungs were excised and the number of nodules was enumerated for each animal without fixation of the lungs. Data were evaluated for statistical significance using a nonparametric unpaired two-tailed *t* test.

RESULTS

Identification of a SA-Le^a Mimic with Higher Antibody Binding Affinity–We previously identified the SA-Le^a peptide mimic DLWDWVVGKPAG from screening a 12-mer peptide combinatorial library using the anti-SA-Le^a specific MAb NS19-9 (45). To analyze amino acid residues that are critical for NS19-9 recognition, an array library of 163 peptides was generated by systematic amino acid replacement in which each position of the starting peptide DLWDWVVGKPAG was replaced by other L-amino acids. In addition, peptides were synthesized with simultaneous incorporation of multiple amino acids or with truncation of specific regions. The ability of MAb NS19-9 to bind substituted peptides within the library was determined after probing of the membrane containing peptide spots with NS19-9 followed by chemiluminescence detection with a peroxidase-labeled anti mouse immunoglobulin G antibody. Spot analysis revealed a distinct pattern of key residues important for binding and, therefore, sensitive to substitution while other residues tolerated replacement by a variety of amino acids (Fig. 1). Comparison of the signal intensities of the array scan revealed that the critical residues for binding were clearly identified within the N-terminal half of the DLWDWVVGKPAG peptide as determined by the lack of antibody binding to substituted peptides (Table IA). In contrast, most of the substitutions within the C-terminus were tolerated (amino acids 6 to 12), not influencing MAb binding. These results indicate that the N-terminus is clearly involved in specific interaction with NS19-9.

Most substitutions of residues 2 to 5 abolished NS19-9 binding, with Trp3 and Trp5 being the most critical. Identical sets of amino acids (His, Tyr, Ala, Asp, Glu, Lys, Arg, Ser) were shown to completely abolish antibody binding while Met significantly decreased the signal upon replacement of either of these residues (Trp 3 and 5). Similarly, substitutions of Leu2 with Ala, Asp, Tyr, Glu, Lys, Arg, and Ser as well as substitution of Asp4 with Glu, Ser, Pro, Val, Met, and Tyr completely abolished MAb binding. All peptides generated by truncation of amino acids 2 to 8 from the N-terminal from the peptide were no longer recognized by the antibody. Furthermore, when the 2-6 amino acid segments covering the key residues involved in antibody binding identified by the single amino acid substitutions were incorporated into the peptide, such a replacement always resulted in abolished binding. Most substitutions upstream from position 5 (positions 6-9) allowed for MAb binding with no evident preference for substituted amino acids. Although, substitution analysis failed to identify significant differences in binding as a result of substitution of residues 9-12 within the C-terminal half of the peptide, MAb did not detect truncated peptides within this region implying the importance of the C-terminus for MAb binding.

Comparison of signal intensities on the peptide array revealed that some substitutions led to enhanced NS19-9 binding allowing for identification of several peptides with increased binding affinity to the antibody (Table IB). Improvement of peptide binding was achieved mainly by substitution of residues 5 to 12 within the lead peptide, whereas no amino acid exchange at N-terminus (residues 1-4) led to the increased binding. The replacement of residues with amino acids containing polar groups such as Glu and Asp showed clearly an enhancing effect at the C-terminus but

not the N-terminus. Array analysis failed to reveal significant differences in the binding intensities between the peptides substituted at different positions, suggesting that single substitution at any position in this region with carboxyl groups can enhance the interaction with the MAb binding site. In addition, substitutions with Ile, Ala and Ser also improved MAb binding. Similarly, the simultaneous replacement of several residues with clusters of amino acids upstream from position 6 demonstrated enhanced binding. The highest intensity signal was, however, observed with peptide DLWDEVVGKPAG containing a single substitution at position 5 with Phe.

A distinct pattern of substitutions that led to increased or abolished signal intensities with respect to the C- and N-terminus suggests that the region close to the N-terminus might contribute to the specificity of the interaction with NS19-9. Amino acids close to the C-terminus appear to add significantly to the affinity of ligand binding.

Competition of DLWDEVVGKPAG Peptide with SA-Le^a for MAb Binding–The lead peptide DLWDWVVGKPAG (#4) and the array selected peptide DLWDEVVGKPAG (#44) were synthesized individually and their binding specificities to NS19-9 MAb was assayed by competitive solid phase enzyme linked immunosorbent assay, as shown in Fig. 2. Both peptides inhibited binding of MAb NS19-9 to a solid phase adsorbed cognate carbohydrate antigen SA-Le^a in a dose dependent manner. IC_{50} of the substituted peptide DLWDEVVGKPAG was established at 70 μM whereas the IC_{50} of the lead peptide DLWDWVVGKPAG was 700

μM (Fig. 2). This 10-fold lower IC_{50} value for the array-selected peptide reflects a higher binding affinity of this peptide as compared to the lead peptide. These data suggest the DLWDEVVGKPAG peptide displays a better fit into MAb binding site as compared with the original peptide. No significant binding of MAb NS19-9 was observed with unrelated peptide in the concentration range up to 5 mM (Fig. 2).

Secondary Structure of Peptides Mimicking Carbohydrate–Secondary structure prediction, based on a neutral net algorithm (52), indicated some propensity of both peptides #4, DLWDWVVGKPAG and DLWDEVVGKPAG, #44 to assume extended or helical structures centered at the mid-chain W/FVVG domain (Fig. 3). The presence of β-pleated sheets was further supported by calculations based on the Fasman-Chou probability values (53), although these predictions placed the extended structure closer to the amino termini, with the C-terminal fragments showing reverse-turn conformations. Low-resolution conformational analysis by CD supported these calculations. In water both peptides exhibited negative bands at 201-202 nm indicative of mostly unordered structures, common for medium-sized peptides. However, the slight redshift from the generally observed 197-198 nm band for entirely random coils highlighted the presence of type I (III) β-turns (54). Comparison of the CD of the two peptides assigned an increased contribution of reverse-turns to the conformational equilibrium for peptide #44, based on the minor redshift of both the 197 nm and the 202 nm bands. A negative shoulder, characteristic for turns, replaced the small positive band between 220 and 230 nm (indicative for random peptide structure). In contrast, peptide #44 lacks a negative shoulder between 210 and 220 nm, clearly

present for peptide #4. Peptides and proteins in β-pleated sheets or type II β-turns exhibit negative bands in this wavelength region. Considering the secondary structural prediction, the presence of extended structure maybe more likely than that of type II β-turns.

In the structure-inducing solvent 50% trifluoroethanol both $\pi\pi^*$ bands for both peptides were redshifted, indicating the acquisition of more ordered structures, as expected (data not shown). The 227 nm positive band of the random coils for peptide #4 disappeared, but the 216 nm negative β-pleated sheet band remained intact as did the 228 nm negative shoulder for peptide #44, suggesting the random conformations were merely replaced by the signature structures (i.e., extended for peptide #4, and turn for peptide #44). Nevertheless, the conformational differences may not represent casual correlations to antibody recognition. Both peptides DLWDWVVGKPAG and DLWDFVVGKPAG highlight the functional role played by the aromatic-X-aromatic motif within the peptide. It is possible that these structure types are realized within the antibody-combining site (55). Turn conformations appear to play an important role in E selectin recognition based on structure activity relations of modified Ser-Glu dipeptides that bind to E selectin (41). The increased binding of peptide with substitution of Phe for Trp would suggest that the Phe directly contributes to MAb and that hydrophobic stacking interactions are important for increased antibody binding and consequently antigenic mimicry. This assertion is supported by X ray crystallographic and molecular modeling studies of carbohydrate mimicking peptides (55, 56).

Inhibition of Tumor Metastasis–SA-Le^a appears to mediate the adhesion of many carcinoma tumors to human umbilical vascular endothelial cells in multiple *in vitro* studies. To establish an *in vivo* experimental metastatic model and to investigate SA-Le^a supported adhesion of tumor cells to lung endothelium, B16F10 murine melanoma cells (syngeneic with C57Bl/6 haplotype) were stably transfected with α1-3/4-fucosyltransferase cDNA in order to express SA-Le^a antigen on the tumor cell surface. The B16F10 clone of B16 cell line does not express E-selectin ligands SA-LeX or SA-Le^a as demonstrated by FACS analysis (not shown). FTIII fucosyltransferase is specific for both type 1 and 2 lactoseries oligosaccharide acceptor substrates and thus is capable of synthesizing both SA-Le^a and SA-LeX, respectively. However, the resulting cell line B16F10FTIII appeared to express SA-Le^a but not SA-LeX as assessed by FACS (not shown), suggesting that type 1 but not type 2 acceptors were available within the cells. Thus, the generated cell line made a suitable model to determine the role of SA-Le^a in the metastatic process since the tumor cells devoid of SA-LeX. The tumorigenic dose for the C57Bl/6 syngeneic tumor cells was established by i.v. injection of various numbers of cells. A 1×10^5 dose was chosen for further experiments as countable lung metastases were observed after i.v. injection of 1×10^5 of B16F10FTIII cells expressing SA-Le^a after 21 days.

The role of tumor cell adhesion to vascular EC via E-selectin and its ligand SA-Le^a interaction in metastasis formation was established *in vivo* in two ways. First, to directly assess the role of E-selectin in tumor colonization *in vivo*, we determined the ability of B16F10 murine melanoma cells expressing SA-Le^a to colonize in the lung of E-selectin KO mice in parallel wild-type C57Bl/6 mice (Fig. 4).

Mice of both strains received i.v. injection of 1 X 10^5 B16F10FtIII tumor cells and mice were examined 3 weeks later. Only 20% of E-selectin deficient animals injected with tumor cells developed small numbers of lung metastasis while the rest of the E-selectin KO mice showed no detectable lung tumor nodules. Statistical analysis gave a P values < 0.009 for E-selectin KO as compared to the control group (Fig. 4, *A* and *C*), respectively. Small nodules were observed in a few E-selectin KO mice that developed tumors whereas all animals in the control group developed multiple metastasis and some of them died earlier than 3 weeks. The results demonstrate that lung metastasis of tumor expressing SA-Le^a antigen is completely abrogated in most of the genetically manipulated mice that lack expression of E-selectin, highlighting the critical role of E-selectin in mediating carcinoma metastasis *in vivo*.

To further test the hypothesis that SA-Le^a expression supports adhesion of tumor cells to E-selectin on EC, we tested the inhibitory effect of peptide mimicking SA-Le^a antigenic structure DLWDEVVGKPAG on lung colonization of B16F10FTIII cells. One X 10^5 tumor cells expressing SA-Le^a were admixed with a solution containing 1 mg of the peptide DLWDEVVGKPAG, followed by administration of the mixture to groups of mice. Our previous studies demonstrate that peptides in general show rather short half-life in mouse serum (57). Therefore, in the peptide inhibition studies *in vivo* the peptide was admixed with the tumor cells to sustain the highest transient concentration of peptide at the time of tumor cell arrival into lung capillary system. Animals were euthanized after 21 days following tumor challenge and the number of metastasis was enumerated in each lung whereas, no

metastatic growth was detected in the liver. Administration of the peptide DLWDFVVGKPAG abrogated on average 50% lung colonization of the B16F10FTIII induced tumor nodules developed in control animals; some mice being completely devoid of tumor nodules (Fig. 4*B*). The injection of the peptide 1 h prior to tumor cells did not influence the rate of metastases formation in comparison with the peptide administered together with tumor cells. Animals treated with peptide showed metastases ranging from 0 to 20 per lung (median 9.9), whereas, animals in the control group developed multiple tumor nodules with the number of metastases per mouse ranging from 3 to 40 per lung (median 20.7) (Fig. 4, *B* and *A*), respectively. The difference was highly statistically significant ($p<0.008$). In addition, B16F10FtIII cells in C57Bl/6 mice developed large tumor masses with diffused infiltration of tumor cells and some mice died before the termination of the experiment (median 16 days). Mice that developed metastases, despite treatment with SA-Le^a mimicking peptide, showed the presence of small tumor nodules and all survived the 3 week observation time. These results suggest that the synthetic structural conformer mimicking SA-Le^a antigen is able to significantly block the adhesion of tumor cells to vascular endothelium at the early stages of the multistep process, thus reducing tumor metastases. This finding strongly suggests that the interaction of SA-Le^a carbohydrate tumor-associated antigen with E-selectin expressed on vascular EC is an important step in establishing tumor metastasis.

DISCUSSION

The histo-blood group related lactoseries antigens are expressed on a variety of human tumor types. In particular SA-Le^a and/or SA-LeX expression on epithelial cancers is correlated with an increased risk of metastasis and poor prognosis (6). Since both neutrophils and carcinoma cells express ligands for E-selectin, it is attractive to hypothesize that early steps of metastatic colonization may be considered equivalent to the initiation of inflammatory process. In our study, the identification of peptides mimicking E-selectin ligand, SA-Le^a, inhibiting metastasis of tumor cells expressing this structure and the abrogated tumor growth in E-selectin KO mice provide an evidence that SA-Le^a E-selectin are important in metastasis formation.

Several laboratories have undertaken the development of high-affinity selectin inhibitors for therapy of acute and chronic inflammatory reactions (16-23). For the identification of high-affinity antagonists for tumor cell adhesion and inhibition of metastasis, we are exploring random peptide library display technology. Highly diverse peptide libraries offer many distinct advantages over difficult chemical or enzymatic synthesis of complex carbohydrates, providing a notably inexpensive and rapid identification and optimization of novel ligands.

SA-Le^a provides the critical adhesion ligand for E-selectin that might facilitate the initial steps involved in a cascade of tumor cell-endothelial interactions leading to metastatic spread. Families of mimics of carcinoma-associated antigen that represent SA-Le^a were identified from a combinatorial peptide library using a MAb specific for

this carbohydrate structure (45). One of the peptides, DLWDWVVGKPAG, was selected and it was established that it specifically competes for binding of MAb for SA-Le^a. This peptide displays an ability to partially inhibit neutrophil recruitment in an acute inflammation model *in vivo* (manuscript in preparation). In the present study this peptide was analyzed by systematic amino acid replacements to identify optimal conformationally stabilized SA-Le^a mimics with higher affinity using a solid phase peptide array library. Comparison of signal intensities revealed significant differences in MAb binding as a result of substitutions, in particular at the N-terminus. Substitution analysis allowed for delineation of key residues that were sensitive to replacement. NS19-9 discriminated against multiple amino acid substitutions affecting its recognition. Specific residues within this peptide were identified that may contribute to the mimicry of carbohydrate structure by the peptide.

On the other hand NS19-9 could tolerate replacement of the lead peptide sequence by a variety of amino acids. These substitutions did not abrogate binding, suggesting that they did not affect the structural specificity required for MAb recognition. This finding further intimates different amino acids in themselves can act as structural mimics within an identified peptide and bind through non-specific interaction. The different consensus sequences among the families of peptides identified with the same MAb from the random peptide library or sequences without an obvious consensus characterized in previous studies support the notion that indeed different amino acid residues provide structural similarity (45). Alternatively, different consensus sequences mimic different topographies of the carbohydrate epitope recognized by the antibody.

The identification of several cross-reactive peptides displaying higher NS19-9 binding further delineate specific residues that may improve upon peptide mimicry of the carbohydrate structure. Several substitutions within the C-terminus, in particular with amino acids containing carboxyl groups, that increased MAb binding were identified suggesting an important role of polar interactions in binding affinity. The strongest signal however resulted from the single amino acid substitution of Phe for the Trp at position 5, creating the peptide DLWDFVVGKPAG.

This peptide was used to explore the extent to which a peptidomimic of a carbohydrate tumor associated antigen is able to reduce the number of experimental metastasis *in vivo*. B16F10FTIII melanoma cells studied in our metastasis model express SA-Le^a carbohydrate antigen and have been observed to form lung tumors after i.v. inoculation through the tail vein. Tumor colonization appears to be highly E-selectin dependent, as the incidence of metastasis was completely abrogated in E-selectin KO mice. The rate of E-selectin was suggested in previous studies where metastases formation *in vivo* was completely or partially abrogated as a result of treatment with E-selectin or E-selectin specific MAb (24-26). However, the initial stages required for tumor colonization are dependent not only on adhesion molecules inducible on endothelial cells, but also on the ligands expressed on tumor cells. The expression of E-selectin ligand, SA-Le^a is likely to contribute to the metastasis of cells expressing this structure. SA-Le^a expressed on B16F10 cells in analogy with our system has been reported to mediate tumor colonization specifically in organs with high expression of E-selectin in E-selectin transgenic mice

(27). The 50% inhibition of tumor metastasis achieved upon administration of the peptide antagonist of SA-Le^a expressed on the B16F10 tumor cell surface in our model can be explained by interruption of the initial steps of cascade of inhibitory events initiated by tumor cell adhesion with this conformational equivalent of the SA-Le^a structure. These findings suggest that expression of SA-Le^a might lead to tumor specific colonization *in vivo*.

Gross histological examination of the lungs (not shown) did not reveal significant differences in the appearance of the small spherical tumor nodules between animals treated with peptide and the control group. This observation suggests that the peptide treatment may indeed block the initial stages of adhesion to lung endothelium required for initiation of tumor cell migration into the subendothelial space (1) resulting in reduced number of nodules but not tumor growth after the micrometastases are established. Although the reduced number of metastases was the prevalent effect of peptide treatment as compared with the control group, large lung tumor masses were observed in untreated animals. The experimental evidence suggests members of all cell adhesion molecule families and carbohydrate structures, SA-Le^a and SA-LeX expression are associated with capillary tube formation and neovascularization necessary to maintain metastasis (58). This might indicate that the anti-angiogenic mechanism of tumor growth inhibition in peptide treated animals also takes place. Similarly, the complete inability of tumor cells colonization in the lungs of E-selectin KO animals may result from the lack of initial adhesion steps mediated by this adhesion molecule as well as impaired angiogenesis in which E-selectin is involved. E-selectin is one of the few adhesion

molecules truly restricted to activated endothelium, thus E-selectin may be used to selectively target activated and/or proliferating endothelium *in vivo* not only by blocking adhesion of tumor cells to EC, but also by halting neovascularization process. Consequently, proliferating microvascular endothelium presents an unique and universal target for anti-cancer therapy, prompting many to investigate biochemical events that constitute potential targets for anti-angiogenic therapy (59).

Our approach demonstrates that peptides mimicking SA-Le^a are able to bind surfaces of proteins specific for these structures and thus they can act as antagonists for the recognition of the cognate carbohydrate antigen or ligand. It is possible that *in vivo* oligosaccharide dependent reduction of metastasis formation may be a function of the interruption of these interactions. Antagonists could interfere with the metastatic process at the level of cellular adhesion and blood vessel formation since E-selectin and SA-LeX are expressed on actively growing blood vessels. Alternatively, peptide mimics may act on signal transduction events mediated by selectins and their ligands and the *in vivo* consequences of selectin-ligand antagonism to the complex signal transduction processes associated with selectin-dependent cell adhesion (60).

While it is unlikely that blocking an adhesion interaction with a single adhesion molecule such as E-selectin will completely inhibit inflammation particularly in chronic disease or metastatic spread, a substantial reduction in pathology is achieved upon 30% reduction of neutrophil recruitment in various inflammatory model systems (17). We suggest by analogy with leukocyte adhesion,

that the interaction of tumor cells with EC represent an initiating event of a cascade of interactions leading to transmigration into the subendothelial space, establishing metastasis implying that this interaction is essential for triggering events necessary for tumor cell migration. Although carbohydrate mimicking peptides, in particular in a monovalent form, are unlikely to be optimal for the treatment of metastatic cancer, the data confirm the importance of carbohydrate ligand SA-Le^a in establishing metastasis. Peptides are frequently considered inferior pharmacophores due to high N to C ratio and low stability in mammalian sera. The latter problem is currently being addressed in our laboratories by serum stability assays. The incorporation of unnatural amino acids or inter-residue bonds at the termini, the most frequent peptide degradation sites, may improve the pharmacological properties of the lead peptide without loss of the binding efficacy. We expect that the use of additional combinatorial synthetic chemistry technologies will allow for improved antagonists of tumor cells adhesion, leading to the further development of agents with greatly enhanced therapeutic potential. Our results demonstrate that using a combinatorial approach based on functional equivalence of chemically dissimilar molecules sharing common surface topology instead of derivatized parental structures is effective in developing antagonists of physiologically important molecular interactions. The important considerations in particular for the development of reagents to interfere with protein-carbohydrate interactions of endothelial cells is to design molecules with higher binding affinity that will be efficient in interrupting this interaction. The presentation of receptor antagonists in multivalent form should render them considerably more potent as adherence

blockers. Peptide multivalency should result in higher affinity binding as compared to low affinity interaction with monovalent peptides and in effect increased peptide/EC interaction and lower concentration of multiple antigen peptides needed to block it. The generation of high affinity ligands might require formation of a "clustered oligosaccharide patch" or a "clustered anionic patch" (20) or a combination of polypeptide backbone and modifications such as sulfation (61).

We postulated that interruption of the adhesion to EC with synthetic peptide antagonists has potential as an anti-adhesion therapy in the processes in which cell adhesion plays a critical role, such as inflammation and metastasis. The prospect of finding mimicking peptides of carbohydrate tumor antigens that competitively inhibit carbohydrate-specific receptors will allow for the design of antagonists of E-selectin and other endolectins. We have demonstrated that peptides mimicking carbohydrate antigens retain conformational properties of cognate carbohydrate structures and can block recognition of cells expressing such a ligands *in vivo*. Thereby, they can mediate anti-metastatic function as demonstrated by blocking of experimental metastasis.

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FIG. 1. **Mapping of the amino acid residues within the sequence DLWDWVVGKPAG that are critical for MAb NS19-9 binding.** The peptide sequence was scanned by substitution of each amino acid with other L-amino acids by spot synthesis and resulted peptides were tested for MAb NS19-9 binding or varying number of amino acids was truncated. The number of peptides is 163 (6 raws, 27 spots each). First spot represents lead peptide with amino acid sequence DLWDWVVGKPAG. (*A*) Control membrane after blotting with BSA instead of specific NS19-9 MAb and secondary antibody; (*B*), membrane after immunoblotting with NS19-9 MAb and the secondary antibody.

FIG. 2. **Effect of Trp5 substitution with Phe in peptide DLWDWVVGKPAG (#4) resulting in peptide DLWDFVVGKPAG(#44) on binding of SA-Le^a specific MAb NS19-9.** Constant amounts of MAb were incubated with increasing amounts of peptides and binding of free antibody to carbohydrate SA-Le^a was measured by enzyme linked immunosorbent assay. Results show competitive inhibition of MAb binding to solid phase SA-Le^a polyacrylamide matrix by 12-mer peptides DLWDWVVGKPAG and DLWDFVVGKPAG with respect to the MAb binding without peptide (100% of binding). *Solid triangle*, peptide #44; *solid square*, peptide #4 and *open circle*, unrelated peptide.

FIG. 3. **CD spectra comparing dodecapeptides DLWDWVVGKPAG and DLWDFVVGKPAG (#4 and #44, respectively).** *Solid line*, peptide #4 and *dotted line*, peptide #44. The spectra were recorded at 0.51 mg/ml for both peptides.

FIG. 4. **Inhibition of lung experimental metastases with peptide DLWDEVVGKPAG.** Tumor cells were admixed with the specific or unrelated peptide solution (1 mg per mouse) and animals were inoculated with 1×10^5 B16F10FtIII tumor cells in 200 µl volume of PBS via tail vein. Results are from 4 experiments (5 mice in each group) are shown. Each dot represents enumerated tumor nodules in one lung in experimental group of C57Bl/6 mice treated with the peptide (*panel B*), control group of C57Bl/6 mice treated with unrelated peptide (*panel A*) and E-selectin KO mice of C57Bl/6 background (*panel C*). Statistical analysis using a nonparametric unpaired *t* test gave a two-tailed P values <0.008 and 0.009 for animals treated with peptide and E-selectin KO, respectively, as compared to control group. The horizontal bars represent median values and vertical bars denote standard deviation.

TABLE IA

Amino acid substitutions within peptide D L W D W V V G K P AG that significantly decrease or abolish binding of MAb NS19-9 using peptide array

D L W D W V V G K P AG

D **A** W D W V V G K P AG
D **D** W D W V V G K P AG
D **Y** W D W V V G K P AG
D **E** W D W V V G K P AG
D **K** W D W V V G K P AG
D **R** W D W V V G K P AG
D **S** W D W V V G K P AG

D L **H** D W V V G K P AG
D L **Y** D W V V G K P AG
D L **F** D W V V G K P AG
D L **M** D W V V G K P AG
D L **A** D W V V G K P AG
D L **E** D W V V G K P AG
D L **D** D W V V G K P AG
D L **K** D W V V G K P AG
D L **R** D W V V G K P AG
D L **S** D W V V G K P AG

D L W **E** W V V G K P AG
D L W **S** W V V G K P AG
D L W **P** W V V G K P AG
D L W **V** W V V G K P AG
D L W **M** W V V G K P AG
D L W **Y** W V V G K P AG

D L W D **H** V V G K P AG
D L W D **Y** V V G K P AG
D L W D **M** V V G K P AG
D L W D **A** V V G K P AG
D L W D **D** V V G K P AG
D L W D **E** V V G K P AG
D L W D **K** V V G K P AG
D L W D **R** V V G K P AG
D L W D **S** V V G K P AG

D L W D W **L** V G K P AG
D L W D W **Y** V G K P AG
D L W D W **A** V G K P AG
D L W D W **S** V G K P AG

D L W D W V **L** G K P AG
D L W D W V **A** G K P AG
D L W D W V **D** G K P AG

D L W D W V D **C** K P AG
D L W D W V D **P** K P AG
D L W D W V D D **Y** P AG
D L W D W V D D **F** P AG

D L **H E**
D L W **E H L**
L D W E W V V G K P A G
D L **D L**
E I H D W V V G K P AG
D L W **E H L**
L D D D W V V G K P AG
E I H E W V V G K P AG
D L W D **H L L A**
L D D L W V V G K P AG
E I H E H L V G K P AG

W D W V V G K P A G
D W V V G K P A G
W V V G K P A G
V V G K P A G
V G K P A G
G K P A G
K P A G

TABLE IB

Amino acid substitutions within peptide D L W D W V V G K P AG that increase the binding of MAb NS19-9 using peptide array

D L W D **F** V V G K P A G
D L W D W V **I** G K P AG
D L W D W V V **A** K P AG
D L W D W V V **S** K P AG
D L W D W V V **E** K P AG
D L W D W V V **D** K P AG
D L W D W V V G **E** P AG
D L W D W V V G **D** P AG
D L W D W V V G K **E** AG
D L W D W V V G K **D** AG
D L W D W V V G K P **D**G
D L W D W V V G K P A**D**
D L W D W V **KE** K P AG
D L W D W V **L A** K P AG
D L W D W V V G **E D** AG
D L W D W V V G K P **EK**
D L W D W V **KE E** P AG
D L W D W V V G **KDEK**
D L W D W V V G **EDEK**
D L W D W V **KE EDEK**

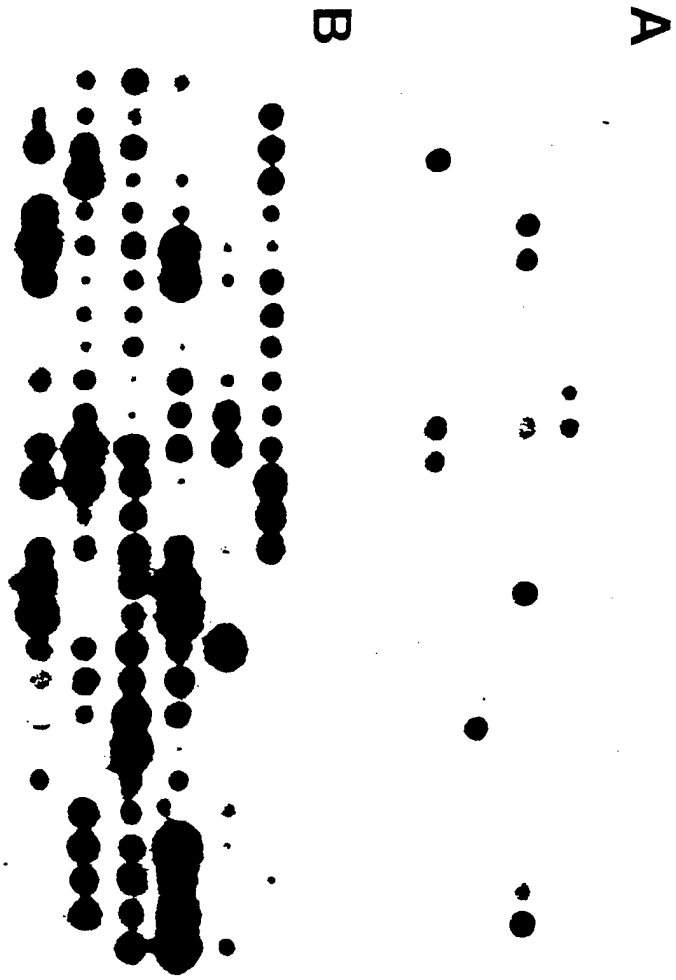

Figure 1

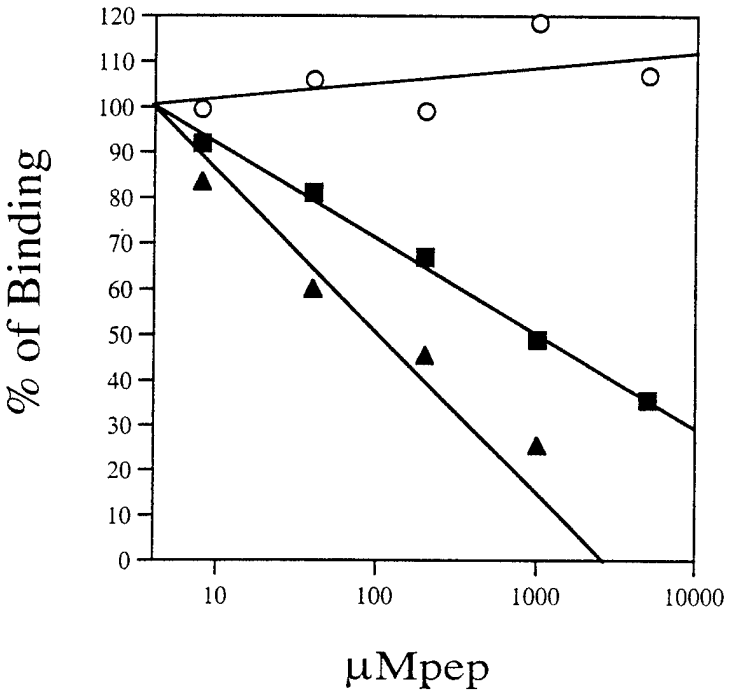
% of Binding
120
110
100
90
80
70
60
50
40
30
20
10
0
10
100
1000
10000
μMpep

Figure 2

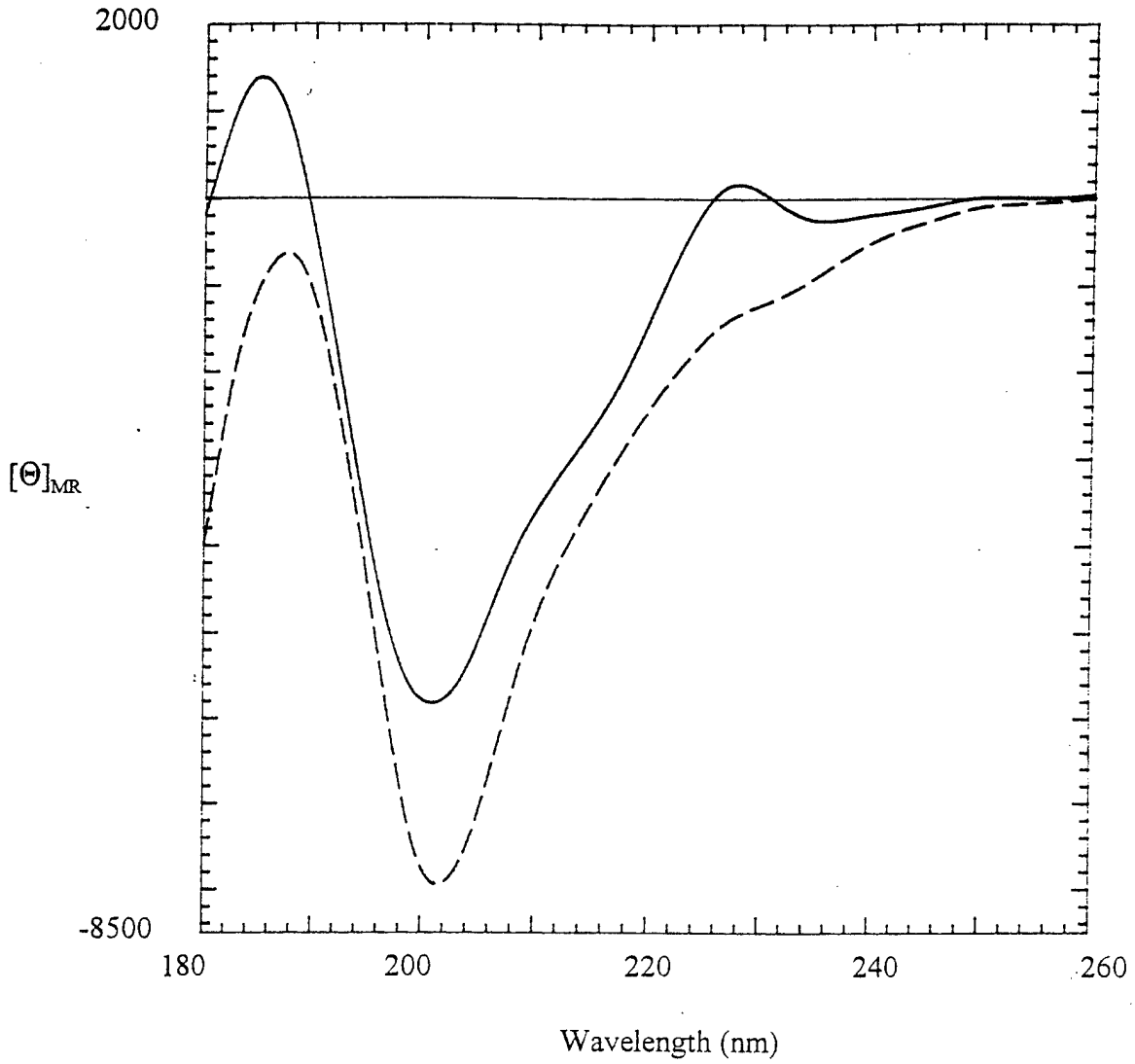

Figure 3

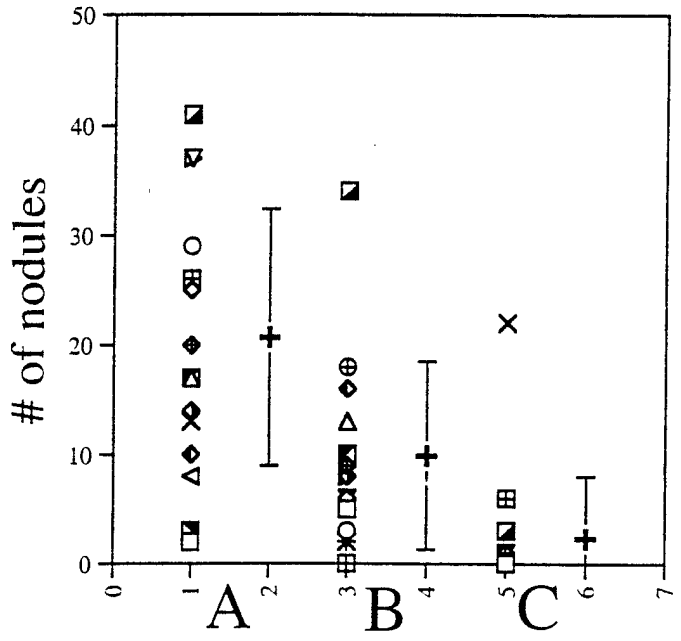

Figure 4